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GENETIC AND PHYSIOLOGICAL CONTROL OF PROTECTIVE ANTIGEN SYNTHESIS BY BACILLUS ANTHRACIS

ANNUAL PROGRESS REPORT AND FINAL REPORT

CURTIS B. THORNE

JULY 1985

Supported by

U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
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The primary objective of the research is to gain information and to develop genetic systems that will contribute to the development of an improved vaccine for anthrax. During the six months covered by this progress report attention was focused on (1) the capsule plasmid, pXO2, of Bacillus anthracis, (2) extending the B. anthracis mating system to include Bacillus subtilis as a participant, and (3) characterization of additional Bacillus thuringiensis conjugative plasmids that are effective in B. anthracis.

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Proof that pXO2 is involved in capsule synthesis came from experiments in which the plasmid was transferred by CP-51-mediated transduction and by a mating system in which plasmid transfer is mediated by a B. thuringiensis fertility plasmid, pXO12. Cells of Bacillus cereus and a previously noncapsulated (pXO2$^-$) strain of B. anthracis produced capsules after the acquisition of pXO2.

Noncapsulated variants of B. anthracis are of two types. One type, which can not revert to capsule-positive, has lost pXO2. The other type, which can revert, has retained pXO2 and presumably has acquired a mutation either on the capsule plasmid or on the chromosome. Results of experiments discussed in this report suggest very strongly that in two examples which were investigated the mutation occurred on the plasmid. When the mutant pXO2 was replaced by wild-type pXO2, the variants acquired the capsule-positive phenotype.

Preliminary results indicate that the fertility plasmid pXO12, which can transfer itself as well as other plasmids within and among strains of B. anthracis, B. cereus, and B. thuringiensis, can also function in B. subtilis. If this result is confirmed and if pXO12 in B. subtilis will engender that strain with the ability to transfer other plasmids to B. anthracis, we will then have a method available for putting the toxin plasmid, pXO1, back into B. anthracis following in vitro manipulations. The fact that B. subtilis is readily transformable with plasmid DNA, while B. anthracis is not, would make such a system extremely useful for further biological characterization of pXO1.

In addition to the B. thuringiensis fertility plasmids, pXO11 and pXO12, which are active in B. anthracis, four new B. thuringiensis fertility plasmids have been found and they are all active in B. anthracis. These have been designated pXO13, pXO14, pXO15, and pXO16. Three of these appear to transfer pXO1 at considerably higher frequencies than those found for transfer of pXO1 in matings with pXO11- or pXO12-containing donors.

This document also includes a Final Report which is a narrative summary of the research carried out during the five-year period of the contract. It is accompanied by pertinent references to specific annual progress reports and other publications.

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SUMMARY

This is an annual progress report together with a final report of research carried out with Bacillus anthracis. Although the report bears the title of annual progress report, it is the last progress report to be submitted under contract DAMD 17-80-C-0099 and covers only the last six-months period (January 1 through July 31, 1985) of the five-year contract. This summary applies only to the six-months report. The section of this document designated Final Report is a narrative summary of the work carried out during the entire five-year period, and it is accompanied by refernces to appropriate annual progress reports and other publications.

The primary objective of the research is to gain information and to develop genetic systems that will contribute to the development of an improved vaccine for anthrax. The toxin-producing, but avirulent, Weybridge (Sterne) strain of B. anthracis is being used. During the past six months we concentrated our efforts on (1) the capsule plasmid of B. anthracis, (2) extending the B. anthracis mating system to include B. subtilis as a participant, and (3) characterization of additional conjugative plasmids of B. thuringiensis that are effective with B. anthracis.

Proof that plasmid pXO2 is involved in capsule synthesis in B. anthracis came from experiments in which the plasmid was transferred by CP-51-mediated transduction and by the mating system in which plasmid transfer is mediated by a B. thuringiensis fertility plasmid, pXO12. Cells of B. cereus and a previously noncapsulated ($pXO2^-$) strain of B. anthracis produced capsules after the acquisition of pXO2 by transduction or by mating. Selection of capsule-positive transcipients following transfer of pXO2 by transduction or mating was facilitated by the use of phage CP-54, which lyses noncapsulated cells but will not adsorb to capsulated cells of either species.

We reported previously that noncapsulated variants derived from the Pasteur strain 6602 are of two types. One type has lost pXO2 and does not revert to capsule-positive, but the other type has retained pXO2 and does revert. Thus, the second type presumably occurs as a result of mutation either on the plasmid or on the chromosome. Two noncapsulated variants of strain 6602 which had retained pXO2 were subsequently cured of the plasmid by treating them with novobiocin. pXO2 was then transferred back into the cured strains by mating them with a strain of B. cereus into which pXO2 had been introduced from a

capsulated strain. Following acquisition of wild-type pXO2, cells of the previously noncapsulated variants displayed the capsule-positive phenotype. These results suggest very strongly that the mutation engendering the capsule-negative phenotype in each of the original rough noncapsulated variants was carried on the plasmid rather than on the chromosome.

Because B. subtilis is readily transformable with plasmid DNA, but B. anthracis is not, it became important to determine whether conditions could be found under which B. subtilis could participate in matings with B. anthracis under the influence of a B. thuringiensis fertility plasmid. If the fertility plasmid, pXO12, for instance, could render B. subtilis fertile and allow it to transfer plasmids to B. anthracis, this would provide a means of putting plasmids that were altered in vitro back into B. anthracis. Results described in this report, although preliminary in nature, suggest that pXO12 does render B. subtilis capable of mating with B. anthracis and transferring the tetracycline resistance plasmid, pBC16, from B. subtilis to B. anthracis. We do not yet know whether plasmids other than pBC16 will transfer in this mating system.

The two B. thuringiensis fertility plasmids, pXO11 and pXO12, which we are currently using for transferring plasmids within and among strains of the three species, B. anthracis, B. cereus, and B. thuringiensis, were derived from B. thuringiensis 4042A subsp. thuringiensis. We have now found that four conjugative plasmids present in other strains of B. thuringiensis are also effective in B. anthracis. These have been designated pXO13, from strain 4049 subsp. morrisoni; pXO14, from strain 4059 subsp. toumanoffi; pXO15, from strain YAL subsp. alesti; and pXO16, from strain BTI subsp. israelensis. Results presented in this report suggest that at least three of these fertility plasmids, pXO13, pXO14, and pXO16, may transfer the B. anthracis toxin plasmid, pXO1, and altered derivatives thereof at frequencies much higher than those found for transfer of pXO1 in matings with pXO11- or pXO12-containing donors.

Foreword

Citation of trade names in this report does not constitute an official Department of the Army endorsement or approval of the use of such items.

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ANNUAL PROGRESS REPORT

This is the sixth annual report together with the final report submitted under contract No. DAMD 17-80-C-0099. Research on the contract began July 1, 1980. The contract was extended for a second year beginning July 1, 1981, for two more years on July 1, 1982, and it was finally extended a third time for a fifth year beginning August 1, 1984. The previous annual reports are dated December 1980, December 1981, December 1982, December 1983, and December 1984. Thus, the present Annual Report covers only the six-month period from January 1 to July 31, 1985.

During the six months represented by this annual report our research concentrated largely on (i) properties and methods of transfer of the capsule plasmid, pXO2; (ii) modification of the B. anthracis mating system to include B. subtilis as a participant; and (iii) characterization of additional B. thuringiensis fertility plasmids effective with B. anthracis. In this report our main efforts for the final six-month period are discussed following a general description of materials and methods. Specific procedures which themselves are results of the research are described as appropriate under individual sections.

Materials and Methods

Organisms. The Weybridge (14) strain of B. anthracis was obtained from the Microbiological Research Establishment, Porton, England in 1957. It was isolated by Sterne (10) and used by the Ministry of Agriculture, Fisheries, and Food (Weybridge, England) as a living spore vaccine. Table 1 lists specific strains and mutants referred to in this report.

Media. For convenience to the reader, compositions of the various culture media referred to in this report are given below. All amounts are for one liter final volume. For preparation of solid medium, 15 grams of agar (Difco) were added per liter of the corresponding broth.

NBY broth: Nutrient broth (Difco), 8 g; Yeast extract (Difco), 3 g.

Phage assay broth: Nutrient broth (Difco), 8 g; NaCl, 5 g; $MgSO_4.7H_2O$, 0.2 g; $MnSO_4.H_2O$, 0.05 g; $CaCl_2.2H_2O$, 0.15 g. The pH was adjusted to 6.0 with HCl.

Phage assay agar: For bottom agar, 15 g of agar were added per liter of phage assay broth. For soft agar, 0.6 g of agar were added per liter.

L broth: Tryptone (Difco), 10 g; Yeast extract (Difco), 5 g; NaCl, 10 g. The pH was adjusted to 7.0 with NaOH.

BHI broth: Brain heart infusion broth (Difco), 37 g.

BHI-glycerol broth: BHI broth with 0.5% (w/v) glycerol added aseptically.

Minimal I: $(NH_4)_2SO_4$, 2 g; KH_2PO_4, 6 g; K_2HPO_4, 14 g; sodium citrate, 1 g; glucose, 5 g; L-glutamic acid, 2 g; $MgSO_4.7H_2O$, 0.2 g; $FeCl_3.6H_2O$, 0.04 g; $MnSO_4.H_2O$, 0.00025 g. The pH was adjusted to 7.0 with NaOH. The glucose and $FeCl_3$ were sterilized separately.

Minimal IC: Minimal I with 5 g of vitamin-free Casamino acids (Difco) and 10 mg of thiamine hydrochloride.

Antisera. All antisera were kindly supplied by personnel of USAMRIID.

Propagation and assay of bacteriophage CP-51 and CP-54. The methods described previously (9, 11) were followed. The indicator for routine assay of CP-51 was B. cereus 569.

Propagation and assay of bacteriophage Wα. Bacteriophage Wα (5) was obtained from B. cereus W (ATCC 11950). It was propagated on B. anthracis 6602 R1 in soft overlays of phage assay agar incubated at 37°C for 17 to 20 hours. It was assayed against the same strain in soft overlays of phage assay agar incubated at 30°C.

Test for capsule production. The ability of B. anthracis and B. cereus to produce capsules was determined by growing cells on R agar (8) or on NBY agar supplemented with 0.7% (w/v) sodium bicarbonate and 10% (v/v) horse serum. Plates were incubated in the presence of 20% CO_2 at 37°C for 24 to 48 hours.

Isolation of spontaneous rough mutants of B. anthracis 6602. Cells were plated for single colonies on NBY agar containing 0.7% (w/v) bicarbonate and 10% (v/v) horse serum and incubated at 37°C in 20% CO_2. After several days rough outgrowth appeared at the edge of some of the mucoid colonies. These were picked and purified by streaking on fresh plates of the same medium.

Isolation of capsulated revertants of rough mutants. To demonstrate

reversion, approximately 1 x 10^5 spores of a rough mutant were spread with 1 x 10^8 PFU of phage Wα on N3Y agar containing bicarbonate and horse serum as above. The plates were incubated at 37°C in 20% CO_2 for two days and examined for mucoid colonies. The presence of capsules was confirmed by phase microscopy.

Procedures used in mating experiments. Cells for mating were grown in 250-ml Erlenmeyer flasks containing 25 ml of BHI broth and incubated at 30°C with slow shaking. Donor and recipient strains were grown separately for 8 to 10 hours from 1% (v/v) transfers of 14- to 15-hour cultures. Each culture was diluted 1:50 in BHI broth, yielding 10^6 to 10^7 cells per ml, and mating mixtures were prepared by mixing 1 ml of donor cells with 1 ml of recipient cells in 20-mm culture tubes. Control tubes contained 1 ml of BHI broth and 1 ml of donor or recipient cells. Mixtures were incubated at 30°C with slow shaking. Samples were removed at times indicated and plated on appropriate selective media for determining the numbers of donors, recipients, and transcipients. Dilutions were made in peptone diluent. Plates were incubated at 30°C and colonies were scored after 24 to 48 hours.

When mating mixtures were prepared with streptomycin-resistant recipients and tetracycline-resistant donors, tetracycline-resistant transcipients were selected on L-agar containing streptomycin (200 µg/ml) and tetracycline (5 or 25 µg/ml. If the recipients were streptomycin-sensitive, tetracycline-resistant transcipients were selected on Min 1C agar supplemented with tetracycline and the appropriate growth requirement of the auxotrophic recipient. For selecting B. cereus transcipients 25 µg of tetracycline per ml was used, but with B. anthracis the number of transcipients recovered was greater when the concentration of tetracycline was only 5 µg per ml. Once transcipients were selected with the lower concentration of tetracycline, they were then fully resistant to 25 µg per ml. When recipients were rifampicin-resistant, rifampicin (10µg/ml) was included in the selection medium.

Transfer frequency is expressed as the number of transcipients per ml divided by the number of donors per ml at the time of sampling. It should be emphasized that the use of both auxotrophic and drug-resistant strains allowed unambiguous strain selection and recognition.

Detection of plasmid DNA. Plasmid DNA was extracted by a modification of the procedure described by Kado and Liu (4). Cells for plasmid extraction were grown in 250-ml Erlenmeyer flasks containing 25 ml of BHI broth supplemented when appropriate with tetracycline (10 µg/ml). With some strains better results

were obtained when 0.5% (w/v) glycerol was included in the BHI broth to prevent sporulation. Cultures were incubated for 16 hours at 37°C on a rotary shaker (100 to 160 rpm). Cells from 25 ml of culture were collected by centrifugation at 10,000 rpm in a Sorvall SS34 rotor for 10 min at 15°C and resuspended in 1 ml of E buffer (0.04 M Tris-OH (Sigma), 0.002 M EDTA (tetrasodium salt, Sigma), 15% sucrose, pH 7.9) by gentle vortexing. Cells were lysed by adding 1 ml of the suspension to 2 ml of lysis buffer prepared by adding 3 gm of sodium dodecyl sulfate and 5 ml of 3 N NaOH to 100 ml of 15% (w/v) sucrose in 0.05 M Tris-OH. The tubes were rapidly inverted 20 times to mix the cells and buffer and they were then held in a 60°C water bath for 30 min. Five-tenths ml of Pronase (Calbiochem-Behring Corp., La Jolla, CA) solution (2 mg per ml in 2 M Tris at pH 7.0) was added, and the tubes were mixed as above and incubated in a 37°C water bath for 20 min. The lysate was extracted with 6 ml of phenol-chloroform (1:1, v/v) by inverting the tubes 40 times. The emulsions were separated by centrifugation at 10,000 rpm for 10 min at 15°C and the aqueous phase was removed for electrophoresis.

Extracts (40 µl) were mixed with 10 µl of tracking dye (0.25% bromphenol blue, 15% ficoll) and samples (40 µl) were applied to horizontal 0.7% agarose (Sigma, Type II medium EEO) gels prepared and run in Tris-borate buffer (0.089 M Tris-OH, 0.089 M boric acid, 0.0025 M EDTA, pH 8.2 to 8.3). Electrophoresis was carried out at 70 V for 90 to 120 min at room temperature. Gels were stained with ethidium bromide (1 µg/ml in Tris-borate buffer).

Screening colonies for protective antigen production. Colonies were picked to plates of R medium (6, 8) and incubated at 37°C in 20% CO_2. Each "halo agar" plate contained 12 ml of R agar plus 2 ml of antiserum prepared in goats by immunization with viable spores of the Sterne strain of B. anthracis. A zone of precipitate formed around colonies that produced protective antigen.

Results and Discussion

Resolution of Problems Associated with Transfer of the Capsule Plasmid pXO2

In the Annual Progress Report dated December 1984 I summarized results of experiments investigating the transfer of pXO2 and demonstrating that the plasmid is associated with capsule formation in B. anthracis. By mating a

$Cap^+Cry^+Tc^r$ donor, i.e., one which harbored the B. thuringiensis fertility plasmid pXO12 in addition to pXO2 and pBC16, with Cap^-Cry^- B. cereus we were able to isolate Cap^+Cry^+ transcipients. Unfortunately, however, we were not able to demonstrate both plasmids, pXO2 and pXO12, in the Cap^+Cry^+ transcipients. Upon electrophoresis of plasmid preparations in agarose gels we found only one intense band which migrated at the rate of pXO12. Because the plasmid band was very intense we reasoned at the time that it was a mixture of pXO2 and pXO12 which did not resolve. We now believe, however, that the plasmid is a recombinant plasmid carrying genes for synthesis of both parasporal crystal and capsule. Its size, based on rate of migration in agarose gels, suggests that it is too small to be a simple cointegrate of pXO2 and pXO12.

The fact that we could not demonstrate unaltered pXO2 in Cap^+ transcipients left us without solid proof that the plasmid is responsible for capsule formation. Theoretically, pXO12 might have incorporated chromosomal genes involved in capsule synthesis. We have now resolved this problem and have shown by the two independent methods described below that acquisition of pXO2 by B. cereus confers upon that organism the Cry^+ phenotype.

Transduction of pXO2 with phage CP-51. Phage CP-51, propagated on B. anthracis 6602, was used to transduce pXO2 into B. cereus 569 UM20-1 Ant^-Str^r and B. anthracis Weybridge A UM23C1 $(pXO1)^-$ $(pXO2)^-$ Ura^-. Phage CP-54, which is active on both B. cereus and B. anthracis, lyses noncapsulated cells but does not adsorb to capsulated cells (C. B. Thorne, unpublished). Its application to transduction plates thus allowed the selection of Cap^+ transductants from a large population of Cap^- cells. The frequency of Cap^+ transductants was approximately 1×10^{-8} per PFU. Plasmid analysis, following clonal purification of Cap^+ transductants, revealed the presence of pXO2, which migrated in agarose gels at the same rate as pXO2 from strain 6602 (Fig. 1). The $pXO2^+$ B. cereus and B. anthracis transductants retained their respective auxotrophic markers, allowing positive identification. They produced capsules when grown in 20% CO_2 on agar containing bicarbonate. Under such conditions the mucoid colonies could not be distinguished from those of encapsulated cells of strain 6602. In the absence of bicarbonate and CO_2, colonies were rough and could not be distinguished from colonies of $pXO2^-$ strains.

The size of pXO2, estimated to have a molecular mass of about 60 megadaltons based on its rate of migration in agarose gels, is probably very close to the maximum size of DNA that can be packaged by CP-51. Previous

results gave a value of approximately 60 megadaltons for the molecular mass of CP-51 DNA (15).

Transfer of pXO2 by Mating. Further experiments on transfer of pXO2 by mating have shown that the results we obtained previously (13), in which capsule genes were apparently transferred on a recombinant plasmid, arose from the use of an aberrant donor (B. anthracis 6602 tr172B-2). This Cap^+Cry^+ 6602 donor strain, which was used to transfer capsule genes to B. cereus and B. anthracis, is aberrant in that it carries what appears to be a recombinant plasmid as discussed above. In more recent experiments we have demonstrated that the majority of Cap^+Cry^+ transcipients of strains 6602 and 4229 carry both plasmids (pXO2 and pXO12) rather than a recombinant plasmid. When they are used as donors in matings with B. cereus, the majority of Cap^+ transcipients inherit pXO2 which migrates in agarose gels at the same rate as pXO2 from strain 6602 or 4229 and which can be distinguished from the fertility plasmid, pXO12.

We improved our method of selecting Cry^+Tc^r transcipients of strains 6602 and 4229. In earlier experiments such transcipients were selected from matings in which the donor was Cry^+Tc^r and either Trp^- or Ura^- by plating mating mixtures on a hydrolyzed casein medium containing tetracycline. Although the donors were Trp^- or Ura^-, they produced a very heavy background of growth as a result of cross feeding and this interfered with isolation of the transcipients. To alleviate this problem, we used nalidixic acid-resistant mutants of 6602 and 4229 as recipients and selected Tc^r transcipients on nutrient broth-yeast extract agar (NBY) supplemented with tetracycline, nalidixic acid, and $MnSO_4$. The $MnSO_4$ was added to increase sporulation (and consequently, parasporal crystal formation) so that Cry^+Tc^r transcipients, i.e., those inheriting the fertility plasmid, pXO12, could be more readily identified. [We had observed that the Pasteur strains, particularly 6602, sporulate very poorly on nutrient agar or NBY agar. The addition of $MnSO_4.H_2O$ (25 mg/liter) results in a dramatic increase in frequency of sporulation].

With the improved method of selecting Cry^+Tc^r transcipients of strains 6602 and 4229, we were able to detect them at a much higher frequency. For example, when B. cereus transcipient, 569 UM20-1 tr 202-1, which inherited both pXO12 and pBC16, was mated with B. anthracis 4229 UM12 Nal^r, and Tc^r transcipients were selected on NBY agar containing tetracycline (5 µg/ml) and nalidixic acid (30 µg/ml), approximately 50% of the Tc^r transcipients also inherited the fertility plasmid, pXO12. One of these, B. anthracis 4229-UM12 tr299-3(pXO2, pXO12,

pBC16), was used as the donor for transferring pXO2 to B. cereus 569 UM20-1. Mating mixtures were plated on NBY agar supplemented with bicarbonate, horse serum, streptomycin and tetracycline. The plates were incubated in 20% CO_2. After 2 or 3 h, 0.1 ml of phage CP-54 (1×10^8 PFU) was spread on some of the plates to lyse Cap^- Tc^r transcipients and allow selection of Cap^+ Tc^r transcipients. Other plates were left untreated so that the total number of Tc^r transcipients could be scored. Incubation was continued for 24 h.

Mating mixtures yielded from 1×10^4 to 1×10^5 Tc^r transcipients per ml, and an average of one Tc^r colony out of 500 was mucoid and produced Cap^+ cells on NBY agar plates containing bicarbonate and serum and incubated in CO_2. Plasmid analysis of 16 or more B. cereus Cap^+ Cry^+ Tc^r transcipients confirmed the presence of pXO2 along with pXO12 and pBC16. Examples are shown in Fig. 2. The Ant^- marker of the original B. cereus recipient was retained, and sporulating cells contained parasporal crystals characteristic of $(pXO12)^+$ cells. With respect to capsule formation, B. cereus cells that received pXO2 by mating could not be distinguished from those that received pXO2 by transduction. As with B. anthracis strains 6602 and 4229, capsules were produced by B. cereus only when cells carrying pXO2 were grown in CO_2.

Nature of Reverting Capsule-negative Mutants of B. anthracis

When Cap^+ strains of B. anthracis are grown under conditions suitable for capsule formation, i.e., on agar containing serum and/or bicarbonate and incubated in 20% CO_2, rough sectors or edges occur frequently among the mucoid colonies. We reported previously (13) that rough variants (Cap^-) derived from the Pasteur strain ATCC 6602 are of two types. One type has lost pXO2 and does not revert to Cap^+. The other type has retained pXO2 and reverts to Cap^+. Thus, the second type presumably occurs as a result of mutation rather than plasmid loss. There is a question, however, as to whether the mutation is on the plasmid or the chromosome. It is conceivable that a mutation on either the plasmid or the chromosome could render a cell defective in capsule formation. Even if one or more genes for capsule formation reside on the plasmid, it is conceivable that a mutation on the chromosome could prevent capsule formation by, for instance, preventing synthesis of a precursor of capsular material. The reverse could also be true.

To investigate whether the mutation in a rough variant that retains pXO2 is

on the chromosome or on the plasmid, we have carried out the following experiments. From three rough variants that harbored pXO2, 6602 R4, R5, and R6, we isolated strains designated R4C1, R5C1, and R6C1 that were cured of the plasmid. We used novobiocin as the curing agent as described in Green, et al. (3). We then mated the cured strains with B. cereus 569 UM20-1 tr60G-10(pXO12::pXO2, pBC16) Cap^+ Cry^+ Tc^r, which carries a cointegrate or recombinant plasmid encoding parasporal crystal synthesis and capsule formation (13). This doror transfers the cointegrate or recombinant plasmid at a relatively high frequency with the result that a significant proportion of tetracycline-resistant transcipients are both Cap^+ and Cry^+.

Cap^+ Cry^+ transcipients were found when 6602 R4C1 and R5C1 were the recipients, and plasmid analysis showed the presence of a plasmid which migrated in agarose gels at the same rate as the cointegrate or recombinant plasmid carried by the donor. These results suggest very strongly that the mutation engendering the Cap^- phenotype in each of the original rough variants, 6602 R4 and R5, was carried on the plasmid and not on the chromosome.

Participation of Bacillus subtilis in the B. anthracis Mating System

Because B. subtilis is readily transformable with plasmid DNA, it became of interest to test whether it could participate in matings with B. anthracis under the influence of the fertility plasmid, pXO12. We reasoned that if pXO12 could render B. subtilis fertile and allow it to transfer plasmids to B. anthracis, this would provide a means of putting plasmids that were altered in vitro back into B. anthracis. We have now shown that B. subtilis can participate in pXO12-directed matings with B. anthracis and some of the results are summarized below.

We first tested B. subtilis strain 168 Ind^- as a recipient in broth matings with B. anthracis, B. cereus, and B. thuringiensis donors, each carrying pXO12 and pBC16. The standard procedure as used for broth matings among B. anthracis, B. cereus, and B. thuringiensis was used and selection was for tetracycline-resistant B. subtilis transcipients ($pBC16^+$). In these tests B. subtilis was not effective as a recipient; no Tc^r transcipients were detected.

We then tested B. subtilis 168 Ind^- as a recipient with each of the same three donors when matings were carried out on membrane filters. In these tests

we obtained Tc^r *B. subtilis* transcipients with *B. anthracis* as the donor. We were very surprised to find that only *B. anthracis* and not *B. cereus* or *B. thuringiensis* would mate with *B. subtilis* under these conditions. When a number of Tc^r *B. subtilis* transcipients were analyzed for their plasmid content, pBC16 was found in all of them, but pXO12 appeared to be absent. We therefore concluded that pXO12 was not transferred to *B. subtilis* or, if it was transferred, it was not maintained.

Assuming that *B. subtilis* could maintain pXO12 once it was introduced into the cell, we then transformed the plasmid into strain 168 Ind^-. Because pXO12 does not carry any selectable markers, we had to rely on cotransformation with pXO12 and pBC16. We transformed with a mixture of pXO12 and pBC16 DNAs and selected Tc^r transformants. These were then screened for parasporal crystal formation as an indicator for the presence of pXO12.

Plasmid analysis of Ind^- Cry^+ Tc^r *B. subtilis* transformants by our modified procedure of Kado and Liu (4), as described under Materials and Methods, revealed the presence of pBC16 but not pXO12. This suggested that (a) pXO12, or at least the crystal gene(s) of pXO12, was integrated into the *B. subtilis* chromosome or (b) our procedure for plasmid extraction was not effective for large plasmids in *B. subtilis*. Based on the results below, we now believe that the latter possibility is the more likely of the two.

When a Cry^+ Tc^r *B. subtilis* transformant was mated with *B. anthracis* strain 6602 by the membrane filter technique, Tc^r transcipients were obtained and approximately 80% of them were Cry^+. Plasmid analysis revealed that these 6602 transcipients contained both pBC16 and pXO12, and mating tests with representative transcipients showed that they were effective donors. Thus, these results suggest that the *B. subtilis* Cry^+ Tc^r transformant carries pXO12 intact and that the strain can transfer both pXO12 and pBC16 to *B. anthracis*.

The next question to be answered is whether pXO12 can mobilize other plasmids that might be transformed into *B. subtilis*. We are currently investigating this using plasmid pTV1, a temperature-sensitive *B. subtilis* plasmid which carries Tn917. At this date we have transformed pTV1 into the fertile Cry^+ Tc^r *B. subtilis* 168 transformant. However, we do not yet know whether (a) pXO12 and pTV1 can be maintained simultaneously in *B. subtilis* or (b) pXO12 can mobilize pTV1. If pXO12 can mobilize pTV1 and cause its transfer to *B. anthracis*, we will then be in a good position to study Tn917-directed mutagenesis in that organism.

Our study of plasmids in B. subtilis would be greatly facilitated by availability of an adequate method for extracting large plasmids from that organism. We are trying to develop such a method.

Other B. thuringiensis conjugative plasmids active in B. anthracis

When we originally screened twelve strains of B. thuringiensis for their ability to transfer the tetracycline resistance plasmid, pBC16, to B. anthracis and B. cereus, five strains were found to be quite active (2, 12). One of the most active was strain 4042A (subsp. thuringiensis) and until recently most of our work on plasmid transfer by conjugation has been done with that strain. This led to the identification of plasmids pXO11 and pXO12 as fertility plasmids capable of transferring themselves as well as other plasmids among members of the three species, B. anthracis, B. cereus, and B. thuringiensis.

The four other strains which were found to be active during the original screening tests were 4049 subsp. morrisoni, 4059 subsp. toumanoffi, YAL subsp. alesti, and BTI subsp. israelensis. We have recently begun investigating the conjugative plasmids in these strains with the interesting result that some of them apparently are capable of promoting the transfer of the B. anthracis toxin plasmid, pXO1, at frequencies considerably higher than those characteristic of pXO11- or pXO12-mediated transfer of pXO1.

Transfer of pBC16 from B. thuringiensis strains 4049, 4059, YAL, and BTI to B. anthracis and B. cereus. To confirm our previous results of experiments in which twelve strains of B. thuringiensis were screened for ability to transfer pBC16 to B. anthracis and B. cereus, we retested the four which, in addition to strains 4042A, gave positive results. Let it be recalled that pBC16 was introduced into the potential donor strains by transduction with phage CP-51 (9, 12). The results of these tests are give in Table 2. Each of the four strains was active in transferring pBC16 to both recipient strains. In general B. cereus gave more transcipients than B. anthracis, a result similar to those obtained with pXO11 and XO12 donors.

Each of these four B. thuringiensis strains contains three to five large plasmids which migrate on agarose gels more slowly than chromosomal DNA. In addition each of the strains contains one or more smaller plasmids which migrate in agarose gels faster than chromosomal DNA. Thus, we had the problem of

sorting out the plasmids to determine which ones were fertility plasmids. Twenty four or more primary transcipients obtained from each of a series of matings between the four B. thuringiensis donors and B. cereus 569 UM20-1 were purified and examined for their plasmid content by agarose gel electrophoresis. (Transcipients isolated from mating mixtures in which the original B. thuringiensis strain was the donor are referred to as primary transcipients. Secondary transcipients are those derived from matings in which the donors were fertile B. anthracis or B. cereus transcipients harboring a B. thuringiensis fertility plasmid.) The results of plasmid analyses suggested that from each of the B. thuringiensis donors one particular high molecular weight plasmid was transferred to B. cereus at a higher frequency than the other plasmids, which appeared to be transferred more or less randomly. This was accepted as tentative evidence that each of the four plasmids which were transferred at high frequencies was a fertility plasmid. The suspected fertility plasmids from strains 4049, 4059, YAL, and BTI were named pXO13, pXO14, pXO15, and pXO16, respectively.

Having tentatively identified the principal self-transmissible plasmids in conjugations with B. cereus, we then examined primary transcipients isolated from mating each of the four B. thuringiensis donors with B. anthracis. The results were similar to those obtained from the mating with B. cereus. The same four plasmids that were identified as probable conjugative plasmids in the B. cereus mating were also found in the B. anthracis matings. Further evidence that each of the four plasmids is a conjugative plasmid was obtained from matings in which it was shown that B. anthracis or B. cereus transcipients harboring any one of the four plasmids were, in turn, able to act as donors.

Plasmid extracts of the four B. thuringiensis donors were subjected to agarose gel electrophoresis along with a plasmid extract of B. thuringiensis 4042A UM8 td2(pXO11, pXO12) to compare the mobilities of pXO13, pXO14, pXO15, and pXO16 with those of pXO11 and pXO12. Based on rates of migration it appeared that pXO13, pXO15, and pXO16 were different from both pXO11 and pXO12, but that pXO14 had a mobility very close if not identical to that of pXO11. Whether or not the two plasmids are identical will be determined by restriction analyses to be done in the future.

Isolation of strains containing only conjugative plasmids. To show that pXO13, pXO14, pXO15, and pXO16 are unquestionably self-transmissible, it was necessary to demonstrate that each of these plasmids alone codes for

mobilization functions as well as the functions determining the cellular mechanisms required for conjugation. Since most of the previously isolated transcipient strains contained lower molecular weight plasmids originating in B. thuringiensis in addition to the large presumed self-transmissible plasmid, it seemed possible, although not probable, that the former encoded certain conjugative functions. Thus to prove conclusively that each of the four plasmids was, in fact, self-transmissible, it became necessary to isolate a series of donor strains each of which contained only pBC16 and one of the large plasmids.

Isolation and identification of such strains was accomplished by a series of manipulations involving plasmid curing by growing cells at 42°C or in the presence of novobiocin, testing a large number of colonies for fertility by the replica plating method described below, and analysis of a large number of transcipients. For each of the fertility plasmids, pXO13, pXO14, and pXO16, we were able to isolate a strain of B. anthracis, B. cereus, or B. thuringiensis which carried only pBC16 and the particular conjugative plasmid in question. Table 3 gives the results of mating experiments in which these strains were used as donors of pBC16. From these results we concluded that each of the three large plasmids is, in fact, an independent conjugative plasmid. We have not yet succeeded in the biological isolation of pXO15.

Previous results (2, 12, 13) from my laboratory showed that the conjugative plasmid, pXO12, derived from B. thuringiensis strain 4042A, encodes parasporal crystal formation in addition to fertility functions. pXO11, derived from the same strain, is also a conjugative plasmid but it does not encode crystal formation. Results to date indicate that none of the four newly identified conjugative plasmids, pXO13, pXO14, pXO15, and pXO16, encodes crystal formation.

Replica plating method of screening colonies for fertility. To facilitate testing large numbers of isolated colonies for fertility as reflected in the ability to transfer pBC16 to recipient cells, we developed a screening method for detecting fertile colonies by replica plating. Up to 25 colonies were picked to BHI agar to form a master plate. The master plates were incubated at 30°C for about 16 hours, and the colonies were then replica plated to BHI agar plates that had been spread with 0.1 ml of spores (approximately 10^8) of a streptomycin-resistant recipient. (Plates spread with 0.1 or 0.2 ml of an 8-hour culture of the streptomycin-resistant recipient prepared from a 1% transfer in BHI broth were equally effective). The plates were incubated about

16 hours at 30°C. After this period of mixed growth on non-selective agar medium, the growth was replica plated to selective L agar plates containing tetracycline (25 μg/ml) and streptomycin (200 μg/ml). After overnight incubation at 30°C patches of transcipients appeared on the plates in areas corresponding to the presence of fertile colonies on the master plates.

Mobilization of pXO1 by pXO13, pXO14, and pXO16. The two conjugative plasmids, pXO11 and pXO12, from B. thuringiensis strain 4042A have been shown in previous studies (2, 13) to mobilize pXO1 from B. anthracis. Therefore, we were interested in determining whether the new conjugative plasmids described here could also mobilize pXO1. This was done by mating transcipients of Weybridge UM44-1(pXO1, pBC16) which had inherited one of the conjugative plasmids with a strain that had been cured of pXO1, Weybridge A UM23C1 str-2. Selection was for transcipients that inherited pBC16 (tetracycline-resistant) and a number of these were chosen randomly for plasmid analysis. Among transcipients derived from donors carrying pXO13, pXO14, or pXO16, respectively, 25%, 6%, and 6% were found to inherit pXO1. These frequencies are much greater than those found for the transfer of pXO1 in matings with pXO11- or pXO12-containing donors. Although we have not had time to confirm these high frequencies, it appears from these preliminary results that these plasmids will be more useful than pXO11 or pXO12 for moving pXO1 and variant plasmids derived from it.

pXO15 is not included in the above discussion because there appears to be an incompatibility between it and pXO1; recipients containing pXO1 have not been found to inherit pXO15. If this is indeed the case, pXO15 will not be a useful plasmid for moving pXO1, although it might be useful for moving other plasmids. This apparent incompatibility will be investigated further. It is conceivable that pXO1 and pXO15 are related plasmids.

TABLE 1. Strains used in this study

Strain[a]	Relevant Characteristics[b]	Relevant Plasmids	Origin/reference
B. anthracis			
Weybridge (Sterne)	Avirulent	pXO1	MRE[c]
Weybridge A	Colonial variant of Weybridge	pXO1	C. B. Thorne
Weybridge A UM23	Ura^-	pXO1	UV[d] of Weybridge A
Weybridge A UM23C1	Ura^-, cured of pXO1	None	C. B. Thorne
Weybridge A UM23C1-1	Ura^-Str	None	UV of UM23C1
Weybridge A UM23C1 td10	Ura^-Cap^+	pXO2	td[e] of UM23C1
Weybridge UM44	Ind^-	pXO1	UV of Weybridge
Weybridge UM44-1	Ind^-Str^r	pXO1	UV of UM44
4229 (Pasteur)	Cap^+Tox^-	pXO2	ATCC[f]
4229 UM12	Nal^rCap^+	pXO2	UV of 4229
4229 UM12 tr299-3	$Nal^rCap^+Tc^rCry^+$	pXO2, pXO12, pBC16	This study
6602 (Pasteur)	Cap^+Tox^-	pXO2	ATCC
6602 R1	Cap^-Tox^-, cured of pXO2	None	This study
6602 R4	Cap^-Tox^-	pXO2	This study

TABLE 1 (continued)

6602 R4C1	Cap^-Tox^-, cured of pXO2	None	This study
6602 R5	Cap^-Tox^-	pXO2	This study
6602 R5C1	Cap^-Tox^-, cured of pXO2	None	This study
6602 R6	Cap^-Tox^-	pXO2	This study
6602 R6C1	Cap^-Tox^-, cured of pXO2	None	This study
6602 tr172B-2	$Cap^+Cry^+Tc^r$	pBC16, pXO2::pXO12	(13)
6602 UM2	Nal^rCap^+	pXO2	UV of 6602
B. cereus			
569	wild type		NRRL[g]
569 K1-1	Ura^-		C. B. Thorne
569 UM20	Ant^-		UV of 569
569 UM20-1	Ant^-Str^r		UV of UM20
569 UM20-1 td10	$Ant^-Str^rCap^+$	pXO2	This study
569 UM20-1 tr60G-10	$Ant^-Str^rCap^+Cry^+$	pBC16, pXO2::pXO12	(13)
569 UM20-1 tr115A-20	$Ant^-Str^rTc^r$	pBC16, pXO16	This study
569 UM20-1 tr202-1	$Ant^-Str^rTc^rCry^+$	pXO12, pBC16	This study
569 UM20-1 tr242A-1	$Ant^-Str^rTc^r$	pBC16, pXO13	This study

TABLE 1 (continued)

569 UM20-1 tr300A-1	$Ant^-Str^rTc^r$	pBC16, pXO14	This study
569 UM20-1 tr305-1	$Ant^-Str^rTc^rCry^+Cap^+$	pXO2, pXO12, pBC16	This study
569 UM20-1 tr305-5	$Ant^-Str^rTc^rCry^+Cap^+$	pXO2, pXO12, pBC16	This study
569 Y7	Ade^-		This study
11950	Carries bacteriophage Wa		ATCC
B. subtilis			
168	Ind^-		C. B. Thorne
B. thuringiensis			
4042A	subsp. thuringiensis	pXO11, pXO12	(1)
4042A UM8	Ade^-Cry^+	pXO11, pXO12	UV of 4042A
4042A UM8 td2	$Ade^-Cry^+Tc^r$	pXO11, pXO12, pBC16	C. B. Thorne
4049	subsp. morrisoni	pXO13	NRRL
4049 td1	Tc^r	pBC16, pXO13	This study
4059	subsp. toumanoffi	pXO14	NRRL
4059 td1	Tc^r	pBC16, pXO14	This study
BTI	subsp. israelensis	pXO16	M. deBarjac
BTI td1	Tc^r	pBC16, pXO16	This study

TABLE 1 (concluded)

YAL	subsp. *alesti*	pXO15	A. Yousten
YAL td1	Tc^r	pBC16, pXO15	This study

[a]In these strain designations, tr denotes a transcipient strain and td denotes a transductant obtained by CP-51-mediated transfer of a plasmid.

[b]Abbreviations: Ade, adenine; Ant, anthranilic acid; Cap, synthesis of capsules; Cry, synthesis of parasporal crystals; Ind, indole; Nal^r, nalidixic acid resistant; Str^r, streptomycin resistant; Tc^r, pBC16 encoded tetracycline resistance; Tox, synthesis of anthrax toxin; Ura, uracil.

[c]MRE, Microbiological Research Establishment, Porton, England.

[d]UV, Mutagenesis by UV light (7).

[e]td, CP-51-mediated transduction.

[f]ATCC, American Type Culture Collection.

[g]NRRL, Agricultural Research Service, Northern Regional Research Laboratory, U.S. Department of Agriculture, Peoria, Ill.

TABLE 2. Transfer of pBC16 from *B. thuringiensis* donors in mating mixtures with *B. anthracis* and *B. cereus* recipients

Donor strain of *B. thuringiensis*	Tc^r transcipients per ml when mated with[a]	
	B. cereus 569 UM20-1 Str^r	*B. anthracis* Weybridge UM44-1 Str^r
4049 td1(pBC16)	1.7×10^5 (4.1×10^{-4})	2.8×10^4 (1.8×10^{-3})
4059 td1(pBC16)	6.2×10^3 (3.3×10^{-5})	1.5×10^3 (1.4×10^{-4})
YAL td1(pBC16)	8.6×10^4 (2.2×10^{-4})	3.8×10^2 (6.3×10^{-6})
BTI td1(pBC16)	1.3×10^3 (6.8×10^{-5})	1.1×10^2 (2.5×10^{-5})

[a]Mating mixtures were incubated 20 h and transcipients were selected on L agar containing 200 µg of streptomycin and 5 µg (for *B. anthracis*) or 25 µg (for *B. cereus*) of tetracycline per ml. Control tubes in which each strain was incubated with 1 ml of BHI broth yielded no spontaneous Tc^rStr^r colonies. The values in parentheses are frequencies, i.e., number of transcipients per recipient cell.

TABLE 3. Mating tests to demonstrate that pXO13, pXO14, and pXO16 are self-transmissible[a]

Donor strain	Tc^r transcipients per ml when mated with *B. cereus*
B. cereus 569 UM20-1 tr242A-1 (pBC16, pXO13) Ant^-	1.4×10^3
B. cereus 569 UM20-1 tr300A-1 (pBC16, pXO14) Ant^-	1.3×10^3
B. cereus 569 UM20-1 tr115A-20 (pBC16, pXO16) Ant^-	1.6×10^5

[a]The recipients were *B. cereus* 569 K1-1 Ura^- or 569 Y7 Ade^-. Mating mixtures were incubated 20 h and transcipients were selected on minimal IC agar containing 25 µg of tetracycline and 40 µg of uracil or adenine per ml. Control tubes in which each strain was incubated with 1 ml of BHI broth yielded no spontaneous Tc^rStr^r colonies. The only plasmids contained in the donors were pBC16 and the indicated fertility plasmids.

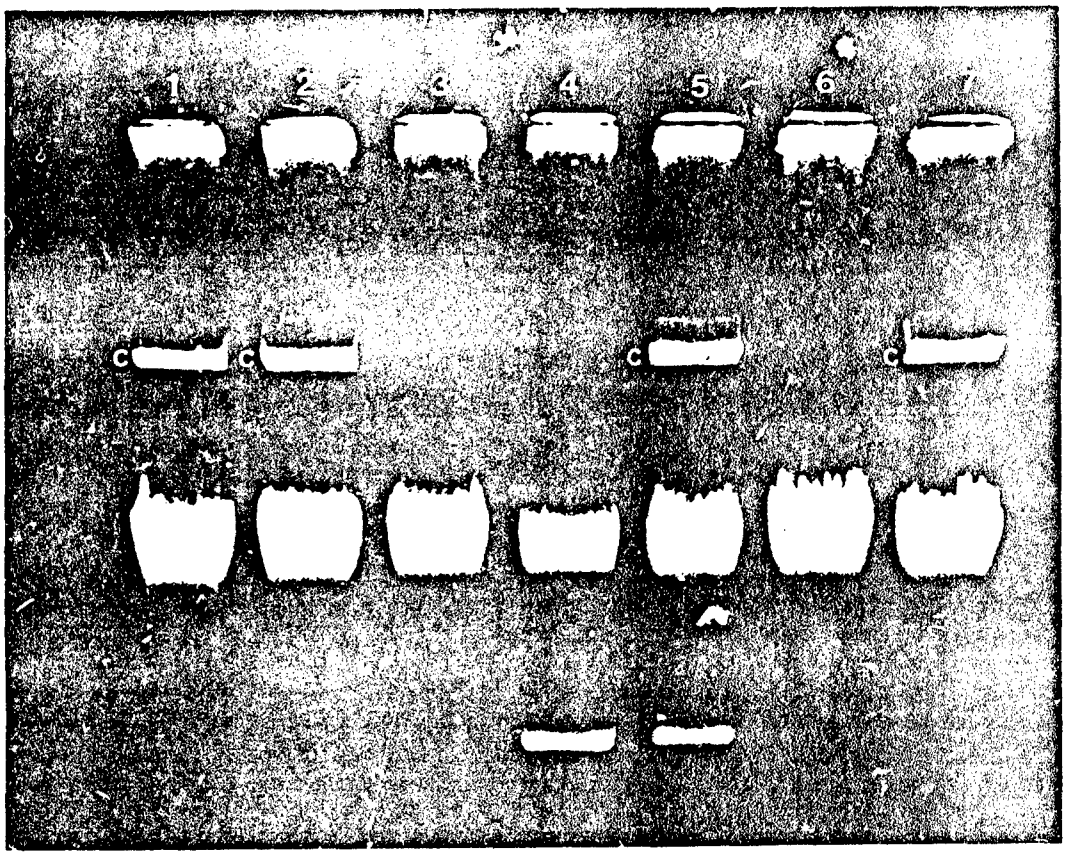

FIG. 1. Agarose gel electrophoresis of plasmid DNA demonstrating transduction of pXO2. Lanes: 1, B. anthracis 6602, Cap^+; 2, B. anthracis 4229, Cap^+; 3, B. anthracis 6602 R1, a Cap^- $(pXO2)^-$ strain; 4, B. cereus 569 UM20-1, a recipient; 5, B. cereus 569 UM20-1 td10, a Cap^+ transductant; 6, B. anthracis Weybridge UM23C1 Cap^-, a recipient; 7, B. anthracis Weybridge UM23C1 td10, a Cap^+ transductant. pXO2 is indicated by the label c. The faint bands above pXO2 are alternative forms of the plasmid.

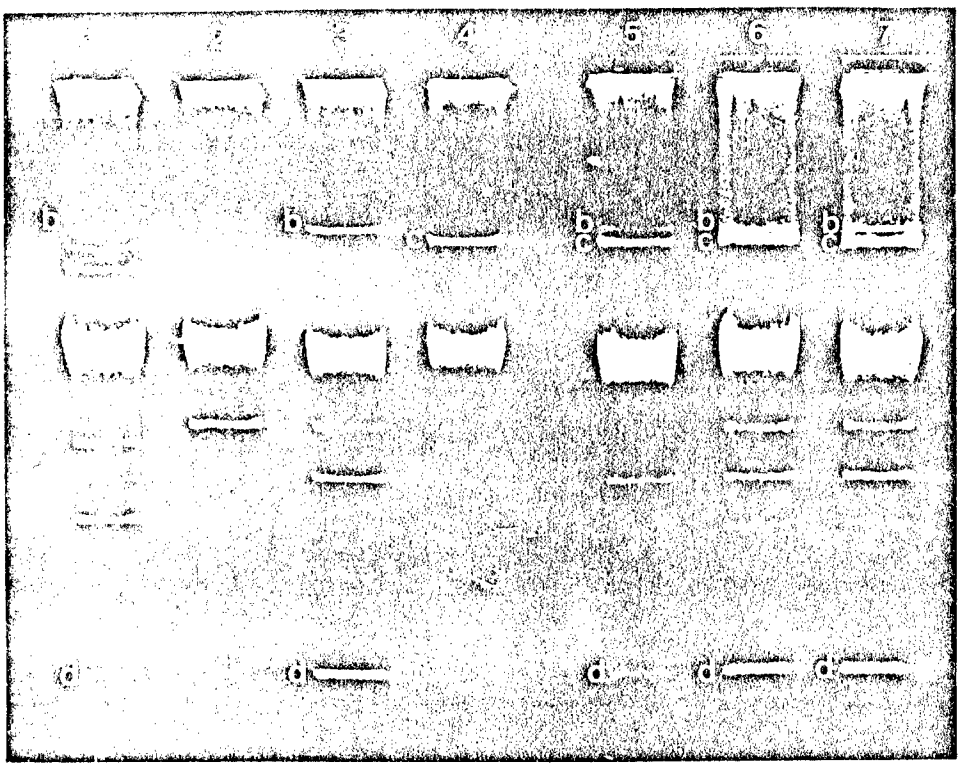

FIG. 2. Agarose gel electrophoresis of plasmid DNA demonstrating transfer of pXO2 and pXO12 by mating. Lanes: 1, B. thuringiensis 4042A UM8 td2; 2, B. cereus 569 UM20-1; 3, B. cereus 569 UM20-1 tr202-1, a Cry^+ Tc^r transcipient derived from mating 4042A UM8 td2 (lane 1) with 569 UM20-1 (lane 2); 4, B. anthracis 4229-UM12 Cap^+; 5, B. anthracis 4229-UM12 tr299-3, a Cry^+ Cap^+ transcipient derived from mating 569 UM20-1 tr202-1 (lane 3) with 4229-UM12 (lane 4); 6 and 7; B. cereus 569 UM20-1 tr305-1 and tr-305-5, respectively, Cry^+ Cap^+ transcipients derived from mating 4229-UM12 tr299-3 (lane 5) with B. cereus 569 UM20-1 (lane 2). Plasmids are labeled as follows: (b) pXO12 (size not determined); (c) pXO2 (approximately 60 megadaltons); (d) pBC16 (2.8 megadaltons).

Publications

The following papers and abstracts have been published during this reporting period:

Papers

Battisti, L., B. D. Green, and C. B. Thorne. 1985. Matins system for transfer of plasmids among Bacillus anthracis, Bacillus cereus, and Bacillus thuringiensis. J. Bacteriol. 162:543-550.

Green, B. D., L. Battisti, T. M. Koehler, C. B. Thorne, and B. E. Ivins. 1985. Demonstration of a capsule plasmid in Bacillus anthracis. Infect. Immun. 49:August.

Thorne, C. B. 1985. Genetics of Bacillus anthracis, p. 56-62. In L. Leive (ed.), Microbiology--1985. American Society for Microbiology, Washington, D.C.

Abstracts

Battisti, L., B. D. Green, and C. B. Thorne. Interspecies plasmid transfer during Bacillus matings. Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, H98, p. 124.

Green, B. D., L. Battisti, and C. B. Thorne. Demonstration of a capsule plasmid in Bacillus anthracis. Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, H99, p. 124.

Ivins, B. E. and C. B. Thorne. Plasmids of Bacillus anthracis. Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, H100, p. 124.

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FINAL REPORT

I. Summary of Research

This is a summary of research on Bacillus anthracis carried out under contract DAMD 17-80-C-0099 during the five-year period July 1, 1980-July 31,1985. Pertinent references to specific Annual Reports or publications in the scientific literature are indicated where appropriate.

The primary objective of the research was to gain information and to develop genetic systems that will contribute to the development of an improved vaccine for anthrax. The Weybridge (Sterne) strain (17) of B. anthracis was used primarily. This is an avirulent strain which is capable of producing toxin but incapable of synthesizing capsule and has been used widely as a live spore vaccine in domestic animals.

Isolation of Mutants and Chromosomal Mapping by Transduction

Weybridge A (17), a variant isolated from the wild-type Weybridge strain, grows well on minimal O medium which contains, in addition to glucose, salts, and glutamic acid, only five other amino acids and thiamine. Minimal O medium therefore serves well as a minimal medium for the isolation of a wide variety of auxotrophic mutants. A number of auxotrophs have been isolated following treatment of spores or cells by ultraviolet light (1) or nitrosoguanidine (4). Our collection of auxotrophic mutants of the Weybridge strain of B. anthracis, all of which were isolated in my laboratory, now includes those requiring indole, tryptophan, valine, leucine, phenylalanine, histidine, arginine, purines (two or three cistrons represented), pyrimidines (at least two cistrons represented), nicotinic acid, biotin, and riboflavin. In addition mutants resistant to a variety of antibiotics have been isolated.

Bacteriophage CP-51, which was isolated in my laboratory several years ago and used for transduction of B. cereus. (15), has been found to be effective for generalized transduction in B. anthracis also (2). Our transduction results have been improved considerably by the use of a temperature-sensitive mutant of CP-51. This mutant does not grow or form

plaques at 42°C, and its efficiency of plating at 37°C, compared to that at 30°C, is very low. By using the temperature-sensitive mutant and selecting transductants at 37°C, we obtained considerably more colonies of transductants (4). Presumably there is much less lysis of potential transductants by the mutant phage than by wild-type phage. By cotransduction with phage CP-51 we have found the following five linkage groups in the Weybridge strain: phe and nic, ura and pyrA, leu and his, ind and trp, and his and pur (4).

Transduction of Plasmids Among Strains of B. anthracis, B. cereus, and B. thuringiensis

We learned that bacteriophage CP-51 can also mediate transduction of plasmid DNA within and among strains of B. anthracis, B. cereus, and B. thuringiensis (2, 14). B. cereus GP7 harbors a 2.8 megadalton multicopy tetracycline resistance plasmid, pBC16. B. thuringiensis 4D11A carries pC194, a 1.8 megadalton multicopy chloramphenicol resistance plasmid. When phage CP-51 was propagated on these strains, it transferred the plasmid-encoded antibiotic resistances to the Weybridge strain of B. anthracis, to B. cereus 569, and to strains of several B. thuringiensis subspecies. The frequency of transfer was as high as 10^{-5} transductants per plaque-forming unit. Tetracycline-resistant and chloramphenicol-resistant transductants contained newly-acquired plasmid DNA having the same molecular weight as that contained in the donor strain. Antibiotic-resistant transductants derived from any of the three species were effective donors of plasmids to recipients from all three species. The development of this method to move plasmids among the three species is one of our most important contributions. For example, it has made possible the use of plasmid markers in experiments on co-curing of plasmids. Furthermore, without the plasmid transduction system we would not have been able to develop the mating system for transfer of plasmids as described later in this report.

Improved Method for Plasmid Analysis

An improved method for analysis of plasmids in strains of B. anthracis, B. cereus, and B. thuringiensis was developed (5). The method, which is based on that of Kado and Liu (10), is much more applicable to these three Bacillus

species than was the original method. Before the availability of this improved method there was not a reliable method for detecting plasmids in cells of these species. This development ranks along with that of plasmid transduction as being one of our most important contributions. Without a good method for plasmid analysis in these species we could not have progressed very far in studying the plasmids of B. anthracis.

The B. anthracis Toxin Plasmid, pXO1

We demonstrated that the Weybridge strain carries a large plasmid which is involved in toxin synthesis (2). [At about the same time the group at the U. S. Army Medical Research Institute of Infectious Diseases also showed that the Sterne strain carries a large plasmid and that strains cured of the plasmid failed to produce toxin (11)]. The plasmid has been designated pXO1. [In earlier reports the plasmid was referred to as pBA1 (2, 3, 4, 13, 18); however, the designation pBA was assigned to another laboratory for naming plasmids and the Plasmid Reference Center at Stanford University has assigned pXO for use by my laboratory. Accordingly, we renamed the plasmid pXO1]. For determining the size of pXO1 purified plasmid DNA isolated from the Weybridge strain was fragmented with HindIII and EcoRI restriction nucleases. The mobilities of the fragments during electrophoresis in agarose gels were compared with those of standard fragments. The size of pXO1 estimated from the HindIII digest was 171.5 kilobase pairs and the size estimated from the EcoRI digest was 177.0 kilobase pairs (5, 12). The average value of 174.3 kilobase pairs is in good agreement with the value of 168.4 plus or minus 7.3 kilobase bairs as estimated by Vodkin and Leppla (18) from electron microscopic measurements.

To determine whether pXO1 might be associated with toxin production and what other effects, if any, it might have on the physiology of the organism, it was necessary to derive cured strains. When the plasmid was first observed, it was cryptic and there was no obvious basis for selecting cured strains. We therefore decided to introduce a drug resistance plasmid into the Weybridge strain to use as a plasmid marker. If such a plasmid were expressed efficiently in the Weybridge strain, and if it persisted in a stable manner in the organism, it would be useful in experiments on co-curing, i.e., we could select variants cured of the drug resistance plasmid and test them for

simultaneous loss of pXO1 by looking at plasmid profiles in lysates of suspected strains. The plasmid we chose for that purpose was pBC16, which was transferred to the Weybridge strain by transduction with phage CP-51 (2, 14).

In curing experiments we used auxotrophic mutants of B. anthracis so that contaminants would not be mistaken for cured strains. The mutants, which also carried pBC16, were grown in the presence of known plasmid curing agents and colonies were screened for tetracycline sensitivity (2). Approximately 10% of colonies tested were sensitive to tetracycline, and gel electrophoresis of lysates showed the absence of pBC16. About 10% of those cured of pBC16 were also cured of pXO1.

Once cured strains of B. anthracis were available, we immediately observed two ways in which they differed from the parent strain carrying pXO1. The cured strains had a colonial morphology different from that of the parent strain, and they sporulated earlier and at a higher frequency than the parent strain. These two observations served as the basis for development of an easier method for isolating cured strains. B. anthracis grows well at 43°C, but it doesn't sporulate well at that temperature. We found that progeny of a good proportion of spores formed at 43°C produced colonies at 37°C that had the altered morphology typical of cured strains. Plasmid analysis showed them to be free of pXO1 (3).

Characteristics of strains cured of pXO1. Strains cured of pXO1 differed from the uncured parent strain in several ways (3). They failed to produce detectable amounts of protective antigen, as determined by an immunodiffusion method (17) in our laboratory, and the group at the U. S. Army Medical Research Institute of Infectious Diseases showed that cured strains also failed to produce lethal factor or edema factor (11). Cured strains also differed from the parent strain in colonial morphology; they sporulated earlier and at higher frequencies than the uncured parent; they grew considerably more poorly than the parent strain on minimal medium; and finally, they were more sensitive than the parent strain to certain bacteriophages.

Although Weybridge A grows well on minimal G medium, derivatives of Weybridge A cured of pXO1 grew very poorly on that medium. Addition of other individual amino acids, purines, pyrimidines, or vitamins did not improve growth of cured strains, although the addition of several amino acids together did result in improved growth. Thus, it appears that although Weybridge A

strains cured of pXO1 have altered growth characteristics in minimal medium, it is very unlikely that loss of the plasmid results in acquisition of specific growth requirements. It seems more likely that loss of the plasmid affects regulatory activities. This difference in growth of cured and uncured strains on minimal medium may be related to the change in sporulation characteristics of cured cells. Cured cells sporulate very early on synthetic medium, and cell yields may be limited by the sporulation process. Asporogenous and oligosporogenous mutants occurred at high frequencies in populations of cured strains and such mutants grew as well as the uncured parent on minimal medium.

Cured cells are more sensitive to three bacteriophages we have tested, CP-51, CP-2, and CP-20. Although the phages grow on both cured and uncured strains, the phage yields produced by cured cells are considerably greater than those produced by the parent strain. Furthermore, under normal conditions used in our laboratory for assay of these phages, they produce distinct plaques in lawns of cured cells but fail to produce visible plaques on lawns of uncured cells.

Transfer of pXO1 by mating. The possibility existed that the curing procedure selected phenotypically changed cells and that some or all of the changes described above were not directly related to loss of plasmid. This seemed unlikely because strains cured by two different procedures, i.e., growing them at high temperatures or growing them in the presence of plasmid curing agents, exhibited the same altered characteristics. However, direct proof that loss of pXO1 was responsible for these changes required that the plasmid be put back into cured strains. We could not transfer pXO1 by CP-51-mediated transduction because the plasmid is too large to be packaged by the phage. With the hope of accomplishing transfer of pXO1, we developed the mating system for transferring plasmids which is described below. We have been successful in transferring pXO1 from donor strains carrying the fertility plasmid, pXO12, to cells of B. anthracis that were previously cured of pXO1 (5, 16). Cured strains that were reinfected with pXO1 by the mating process could not be distinguished from the uncured Weybridge A parent strain from which they were originally derived. This confirms that the phenotypic changes observed in cured strains are consequences of plasmid loss.

We have also transferred pXO1 to B. cereus 569 by the mating system (5). Characteristics of B. cereus harboring the plasmid are currently under

investigation in my laboratory and by Dr. Stephen H. Leppla at USAMRIID.

The B. anthracis Capsule Plasmid, pXO2

We have shown that the Pasteur vaccine strains ATCC 6602 and ATCC 4229 contain a capsule plasmid which has been designated pXO2 (5, 6, 8), and experiments carried out in collaboration with Dr. Bruce Ivins of USAMRIID confirmed that pXO2 is also present in virulent strains of B. anthracis (8, 9). Proof that pXO2 is involved in capsule synthesis came from experiments in which the plasmid was transferred by CP-51-mediated transduction and by the mating system in which plasmid transfer was mediated by the B. thuringiensis fertility plasmid pXO12. Cells of B. cereus and a previously noncapsulated ($pXO2^-$) strain of B. anthracis produced capsules after the acquisition of pXO2 (6, 8). The B. cereus and B. anthracis transductants and transcipients retained their respective auxotrophic markers, allowing positive identification. They produced capsules when grown in 20% CO_2 on agar containing bicarbonate. Under such conditions the mucoid colonies could not be distinguished from those of encapsulated cells of strains 6602 or 4229. In the absence of bicarbonate and CO_2, colonies were rough (noncapsulated) and could not be distinguished from colonies of $pXO2^-$ strains. Plasmid analysis revealed the presence of pXO2, which migrated in agarose gels at the same rate as pXO2 from strains 6602 and 4229. The size of pXO2, estimated to have a molecular mass of about 60 megadaltons, is probably very close to the maximum size of DNA that can be packaged by CP-51. Previous results gave a value of approximately 60 megadaltons for the molecular mass of the CP-51 genophore (19).

We reported previously (5) that noncapsulated variants derived from the Pasteur strain 6602 are of two types. One type has lost pXO2 and does not revert to capsule-positive, but the other type has retained pXO2 and does revert. Thus, the latter type presumably occurs as a result of mutation either on the plasmid or on the chromosome. Two noncapsulated variants of strain 6602 which had retained pXO2 were subsequently cured of the plasmid by treating them with novobiocin. pXO2 was then transferred back into the cured strains by mating them with a strain of B. cereus into which pXO2 had been introduced from an encapsulated strain. Following acquisition of wild-type pXO2, cells of the previously noncapsulated variants displayed the

capsule-positive phenotype. These results (6) suggest very strongly that the mutation conferring the capsule-negative phenotype in each of the original rough noncapsulated variants was carried on the plasmid rather than on the chromosome.

Mating System for Transfer of Plasmids Among B. anthracis, B. cereus, and B. thuringiensis

To facilitate the analysis of genetic determinants carried by large resident plasmids of B. anthracis, a mating system has been developed which promotes plasmid transfer among strains of B. anthracis, B. cereus, and B. thuringiensis. Transfer of the selectable tetracycline resistance plasmid, pBC16, and other plasmids from B. thuringiensis to B. anthracis and B. cereus recipients occurred during mixed incubation in broth. Two plasmids, pXO11 and pXO12, found in B. thuringiensis were responsible for plasmid mobilization. B. anthracis and B. cereus transcipients inheriting either pXO11 or pXO12 were, in turn, effective donors. Transcipients harboring pXO12 were more efficient donors than those harboring pXO11; transfer frequencies ranged from 10^{-4} to 10^{-1} and from 10^{-8} to 10^{-5}, respectively. Cell-to-cell contact was necessary for plasmid transfer and the addition of DNase had no effect. The high frequencies of transfer, along with the fact that cell-free filtrates of donor cultures were ineffective, suggested that the transfer was not phage-mediated. B. anthracis and B. cereus transcipients which inherited pXO12 also acquired the ability to produce parasporal crystals resembling those produced by B. thuringiensis, indicating that pXO12 carries gene(s) involved in crystal formation. Transcipients which inherited pXO11 did not produce crystals. This mating system provides an efficient method for interspecies transfer of a large range of Bacillus plasmids by a conjugation-like process (4, 5, 7).

Other B. thuringiensis conjugative plasmids active in B. anthracis. The two B. thuringiensis fertility plasmids, pXO11 and pXO12, which we are currently using for transferring plasmids within and among strains of the three species, B. anthracis, B. cereus, and B. thuringiensis, were derived from B. thuringiensis 4042A subsp. thuringiensis. We have now found that four conjugative plasmids present in other strains of B. thuringiensis are also effective in B. anthracis. These have been designated pXO13, from strain 4049

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II. List of Publications

Papers

Ruhfel, R. E., N. J. Robillard, and C. B. Thorne. Interspecies transduction of plasmids among Bacillus anthracis, B. cereus, and B. thuringiensis. J. Bacteriol. 157:708-711(1984).

Battisti, L., B. D. Green, and C. B. Thorne. Mating system for transfer of plasmids among Bacillus anthracis, Bacillus cereus, and Bacillus

thuringiensis. J. Bacteriol. 162:543-550(1985).

Thorne, C. B. Genetics of Bacillus anthracis, p. 56-62. In L. Leive (ed.), Microbiology--1985. American Society for Microbiology, Washington, D.C.

Green, B. D., L. Battisti, T. M. Koehler, C. B. Thorne, and B. E. Ivins. Demonstration of a capsule plasmid in Bacillus anthracis. Infect. Immun. 49:August (1985)

Ph. D. Dissertation

Robillard, N. J. Changes associated with plasmid loss in Bacillus anthracis. Ph. D. Dissertation. University of Massachusetts. 1984.

Abstracts

Robillard, N. J., T. M. Koehler, R. Murray, and C. B. Thorne. Effects of plasmid loss on the physiology of Bacillus anthracis. Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, H54, p. 115.

Ruhfel, R. E., N. J. Robillard, and C. B. Thorne. CP-51-mediated interspecies transduction of plasmid pBC16 among Bacillus cereus, Bacillus thuringiensis, and Bacillus anthracis. Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, H76, p. 118.

Battisti, L., B. D. Green, and C. B. Thorne. Interspecies plasmid transfer during Bacillus matings. Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, H98, p. 124.

Green, B. D., L. Battisti, and C. B. Thorne. Demonstration of a capsule plasmid in Bacillus anthracis. Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, H99, p. 124.

Ivins, B. E. and C. B. Thorne. Plasmids of Bacillus anthracis. Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, H100, p. 124.

III. List of Personnel

Principal Investigator, C. B. Thorne, Professor

The following graduate students in pursuit of M. S. and Ph. D. degrees worked on the contract:

Battisti, Laurie
Green, Brian D.
Heemskerk, Deborah
Koehler, Theresa M.
Reddy, Amala
Ruhfel, Robert E.
Robillard, Norman J.

The following were employed as technicians for part of the contract period:

Murray, Richard
Ruhfel, Robert E.

The following undergraduate students were employed on an hourly basis for various periods of time as laboratory workers:

Burke, Diane	Mendelsohn, Cathy
Casella, Patricia	Murray, Richard
Dalrymple, Carol	Price, Bonnie
Freedman, Matthew	Robertson, Lee
Jaworski, Deborah	Thorne, Bradford
Douglas, Cameron	Winnerman, Lynn
McGrath, Brian	Wolfson, Galena

IV. List of Those Who Received Graduate Degrees While Working on the Contract

Robillard, Norman J., Ph. D.
Battisti, Laurie, M. S.
Green, Brian D., M. S.
Koehler, Theresa M., M. S.
Ruhfel, Robert E., M. S.

Each of those listed as having received the M. S. degree is continuing graduate work toward the Ph. D. degree.

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